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Insulin-like Growth Factor Binding Protein Gene Expression in the Pregnant Rat Uterus and Placenta

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While the insulin-like growth factor (IGF) system plays a fundamental role in regulating embryonic and placental growth, the specific contributions of the six IGF binding proteins (IGFBPs 1–6) to these processes are not well understood. We here focus on IGFBP expression in the extraembryonic environment, which both supports and constrains embryonic growth, and have used *in situ* hybridization to determine sites of IGFBP mRNA synthesis in the pregnant rat uterus and placenta. We find that all IGFBPs are expressed in distinct, changing patterns in the uterine endometrium, at the decidual boundary, in the decidual vasculature, and in the myometrium during pregnancy. Within the endometrium, the most prominent change is that expression of IGFBP-1 begins in some, but not all, endometrial glands prior to implantation and then expands to include all secretory epithelia shortly after implantation. During the period of rapid decidual proliferation that follows implantation, IGFBP-3, -4, and -5 transcripts are all detected in a laminar array at the boundary between the decidua and the nondecidualized endometrium. In the decidual vasculature at Day (d) 8.0, both IGFBP-3 and IGFBP-4 mRNAs are detected in dilating blood vessels, with BP-3 most prominent in the antimesometrial plexus and BP-4 primarily at the mesometrial pole. Later (d11.5), all decidual vessels express high levels of IGFBP-3 and lower levels of IGFBP-4 mRNAs. Finally, changes in expression of several IGFBPs also occur within the myometrium during pregnancy. For example, IGFBP-2 is expressed in the inner circular layer shortly after implantation, and expression increases through late gestation. In contrast, IGFBP-5 hybridization occurs over both myometrial layers before implantation, but decreases in intensity and spatial distribution as pregnancy proceeds. Finally, and most strikingly, IGFBP-6 expression, barely detectable in the d7.0 myometrium, gradually increases until it is very strongly transcribed during the placental stages. Taken together, these observations suggest multiple roles for IGFBPs in supporting implantation, regulating the extent of decidualization, modulating local levels of vascular IGFs, and regulating uterine muscular growth.

INTRODUCTION

Several components of the mitogenic insulin-like growth factor (IGF) system are required for normal embryonic and placental growth in rodents (Baker *et al.*, 1993). Specifically, mice unable to produce IGF-II suffer both embryonic and placental growth deficits (DeChiara *et al.*, 1990), while only the embryo is affected in the absence of IGF-I (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993) or the type 1 IGF receptor (IGFR1, see Liu *et al.*, 1993). IGF-I is also essential for normal development of the reproductive systems of both sexes (Baker *et al.*, 1996). For example, in females lacking IGF-I, maturation of the myometrium is prevented and Graafian follicles are absent from the ovaries (Baker *et al.*, 1996).

There are at least three known IGF receptors. The IGFs are thought to exert their activity primarily through IGFR1, but they may also activate the insulin receptor (reviewed in LeRoith *et al.*, 1995b). Genetic evidence suggests that IGF-II also acts through an additional unidentified receptor (IGFRX) in the placenta (Baker *et al.*, 1993; Ludwig *et al.*, 1996). Finally, the bifunctional type 2 IGF receptor/mannose-6-phosphate receptor (M6PR/IGFR2) binds IGF-II, but not IGF-I (Morgan *et al.*, 1987). Recent gene targeting studies have shown that the major IGF-specific function of IGFR2 is the clearance of IGF-II from the extracellular environment (Lau *et al.*, 1994; Wang *et al.*, 1994; Ludwig *et al.*, 1996).

Complicating any physiological analysis of the IGF sys-

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tem is a family of high-affinity IGF binding proteins (IGFBPs) present in most, if not all, body fluids (reviewed in Jones and Clemmons, 1995) and expressed by a wide variety of cell types during embryonic development (Wood *et al.*, 1990, 1992; Pintar *et al.*, 1991; Streck *et al.*, 1992; Cerro *et al.*, 1993; Schuller *et al.*, 1993; Green *et al.*, 1994). IGFBPs bind the IGFs with affinities comparable to, and often greater than, the affinities of IGF receptors for IGFs (Gammeltoft *et al.*, 1985; Kiefer *et al.*, 1993). Further, they account for the majority of cell-surface IGF binding sites in fibroblasts (McCusker *et al.*, 1990). Ascribing specific *in vivo* functions to the IGFBPs has been difficult since, depending on the experimental paradigm *in vitro*, IGFBPs can inhibit or enhance IGF activity and can also act independently of the IGFs (for detailed review, see Jones and Clemmons, 1995). It is clear, however, that IGFBPs can exert tissue-specific effects during development, since recent genetic experiments have revealed that IGFBP-2 is required for normal growth of the spleen (Pintar *et al.*, 1996).

The IGF system has been implicated in several events which are necessary to support embryonic growth and development, such as the rapid growth of the uterus during pregnancy and the differentiation of the decidua from the endometrial stroma (reviewed in Simmen *et al.*, 1995). Also, since maternal, as well as fetal, IGF-I levels influence the growth of the conceptus (Gluckman *et al.*, 1992), uterine IGFBPs may likewise be important regulators of embryonic growth. Several groups have reported changes in maternal serum IGFBP profiles during pregnancy in a variety of species (Davenport *et al.*, 1990; Fielder *et al.*, 1990; Gargosky *et al.*, 1990; Giudice *et al.*, 1990, 1993; Wang *et al.*, 1991; Baxter and Saunders, 1992; Lee *et al.*, 1993; Chard *et al.*, 1994; Markoff *et al.*, 1995; Nason *et al.*, 1996). Likewise, uterine and ovarian IGFBP transcription varies during the estrous cycle (Geisert *et al.*, 1991; Nakatani *et al.*, 1991; Erickson *et al.*, 1992a,b; Simmen *et al.*, 1992; Ghahary *et al.*, 1993; Girvigian *et al.*, 1994), so it is reasonable to expect that changes in uterine IGFBP expression could also occur during pregnancy. To date, little information about the cellular location of IGFBP expression in the pregnant uterus has been available. IGFBP-1, a major endometrial protein in primates (Bell *et al.*, 1985; Fazleabas *et al.*, 1989) and cellular marker of decidualization in humans (Rutanen *et al.*, 1991) is expressed by rat uterine luminal epithelial (ULE) cells during early decidual stages (Sadek *et al.*, 1994). IGFBP-4, generally considered to be an IGF inhibitor (Jones and Clemmons, 1995), is localized to the endometrial stroma (Markoff *et al.*, 1995; Pintar *et al.*, 1996), and later, IGFBP-2 mRNA is prominently expressed by cytotrophoblasts during placentation (Zhou and Bondy, 1992). To address potential effects of IGFBPs on IGF action during pregnancy, we here use *in situ* hybridization to elucidate the expression of all six IGFBP mRNAs in the uterus and placenta through the preimplantation, embryonic, and fetal periods.

MATERIALS AND METHODS

All studies were conducted in accordance with NIH guidelines (Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, 1993). Uteri and placentas from timed-pregnant Sprague–Dawley rats, as well as uteri from late estrous rats, were dissected and fixed in 4% paraformaldehyde/PBS, equilibrated in 20% sucrose/PBS, embedded in O.C.T. (Miles, Inc., Elkhart, IN), sectioned as described previously (Hockfield *et al.*, 1993), and mounted on slides coated with TESPA (Pierce Chemical Company, Rockford, IL). Conceptuses were staged precisely by examining embryonic limb morphology based on the method of Wanek *et al.* (1989). Two uteri at Day (d) 3.5 pc and a minimum of eight concepti from two or more litters each at ages d7.0, d8.0, and d10.0 were used for this study. Two uteroplacentas each from two different litters at each stage were used for the d11.5, d14.5, and d18.5 experiments. For comparison, C57BL/6J mouse concepti at d14.0 and d18.0 were also examined. ^{35}S-labeled antisense cRNAs for the IGFBPs, IGFs, and IGFR1 were transcribed from plasmids pRBP1-501 (Murphy *et al.*, 1990), pG3-2-11 (Brown *et al.*, 1989), pRBP3-AR (Shimasaki *et al.*, 1989), pRBP4-SH (Shimasaki *et al.*, 1990), pGEM3Z/mBP5,2-3 (James *et al.*, 1993), pRBP6-PP (Shimasaki *et al.*, 1991), pRIGF1-ES (Roberts *et al.*, 1987), pRIGF-II-BP (Whitfield *et al.*, 1984), and pRIGFR1-ER (Werner *et al.*, 1989). pRBP1-501 contains a PCR fragment (nucleotides 486–892) of a rat IGFBP-1 cDNA clone. pG3-2-11 contains a *Hin*dIII–*Sac*I fragment (nucleotides 502–1087) of a rat IGFBP-2 cDNA clone. pRBP3-AR contains an *Apa*I–*Rsa*I fragment (nucleotides 163–861) of a rat IGFBP-3 cDNA clone. pRBP4-SH contains a *Sma*I–*Hin*dIII fragment (nucleotides 435–878) of a rat IGFBP-4 cDNA clone. pGEM3Z/mBP5,2-3 contains nucleotides 512–988 of a mouse IGFBP-5 cDNA clone. pRBP6-PP contains a *Pst*I fragment (nucleotides 229–475) of a rat IGFBP-6 cDNA clone. pRIGF1-ES contains a 320-base *Eco*RI/*Sau*3A fragment of a rat IGF-I cDNA clone. pRIGF-II-BP contains a 551-base *Bam*HI–*Pst*I fragment of a rat IGF-II cDNA clone. pRIGFR1-ER contains a 265-base *Eco*RI–*Sma*I fragment of a type 1 IGF receptor rat cDNA clone. Transcriptions were performed using SP6, T7, or T3 RNA polymerases (Promega, Woods Hollow, WI, and Boehringer-Mannheim, Indianapolis, IN) in the presence of ATP, CTP, GTP, and [^{35}S]UTP. The resulting cRNA transcripts were purified on Sephadex G-50 columns (Boehringer-Mannheim) and used without hydrolysis.

In situ hybridizations were performed as previously described (Hockfield *et al.*, 1993). In short, tissue sections were mounted on slides, briefly postfixed in 4% paraformaldehyde/PBS, dehydrated in ethanol series, acetylated with 0.05 *M* triethanolamine/0.25% acetic anhydride (v/v) (Sigma, St. Louis, MO), washed in 0.2× SSC, redehydrated in ethanol series, prehybridization treated at room temperature, and then hybridized overnight at 50°C, with probe concentrations of $\sim 2 \times 10^4$ cpm/μl. Sections were washed in 50% formamide/10 m*M* DTT/1× SSC, treated with 100 μg/ml RNaseA (Boehringer-Mannheim), washed in 0.5× SSC, dehydrated, and subjected to X-ray film or liquid nuclear track emulsion autoradiography (1-, 2-, or 4-week exposures) and hematoxylin/eosin counterstaining. Hybridization with control (sense) RNA yielded very low backgrounds. The distinct hybridization patterns observed in these experiments further demonstrate probe specificity. In all experiments, specimens from several stages were hybridized concurrently, indicating that changes in hybridization pattern and intensity reflect changes in mRNA expression and not artifactual variation between experiments.

Microscopic visualization of sections was accomplished using either dark-field illumination at low magnification or concurrent bright-field and epipolarization illumination at high magnification. Images were recorded by photomicrography or computer-assisted video microscopy. Video images were digitized by a Sony

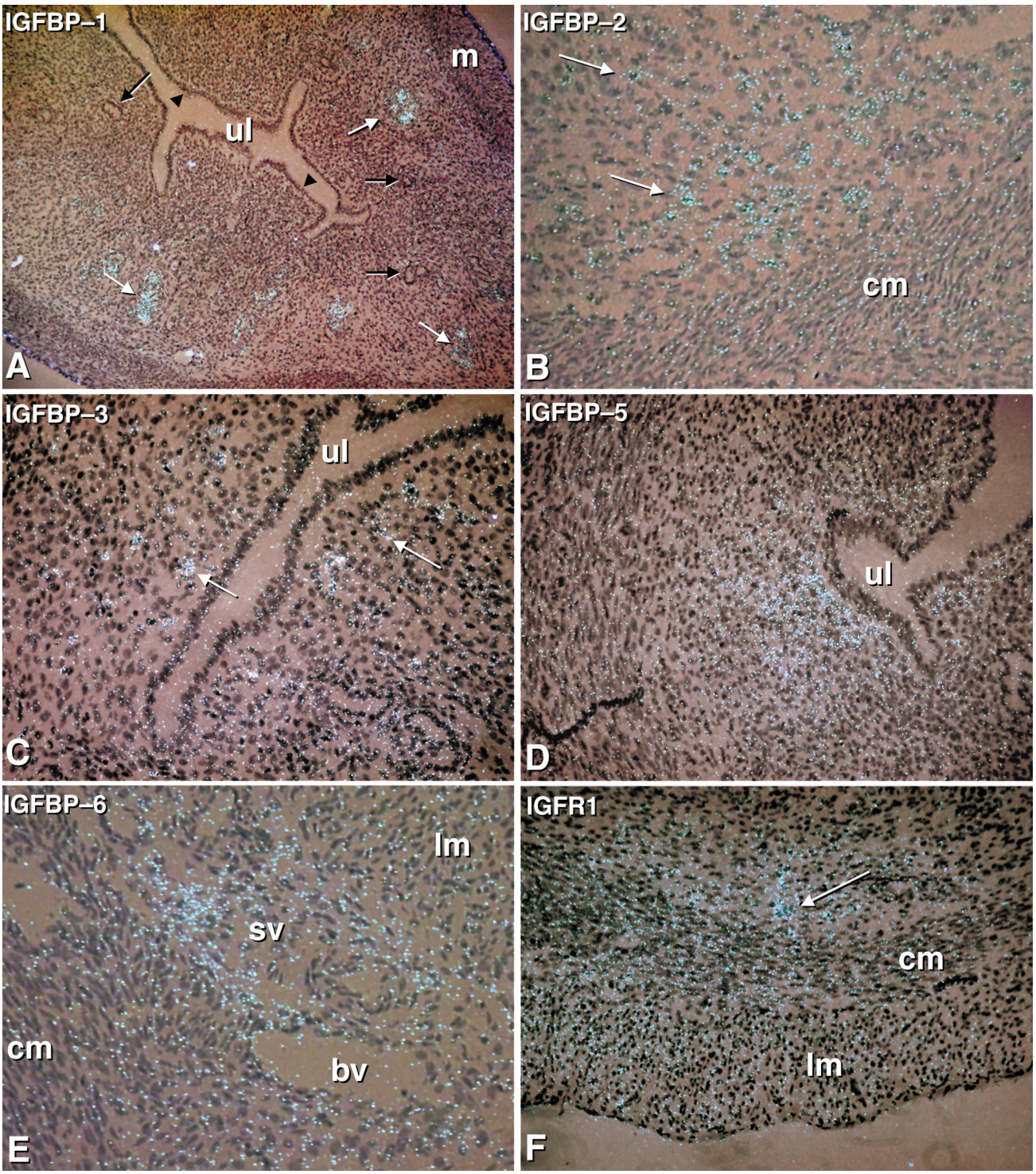


FIG. 1. IGFBP mRNAs are present in the preimplantation uterus. All panels are high-power epipolarization/bright-field (EP/BF) photomicrographs of d3.5 pc uteri, except B and E, which are EP/BF video micrographs of the same material. (A) IGFBP-1 is expressed by some (white arrows), but not all (black arrows), endometrial glands and is absent from the luminal epithelium (arrowheads); 100×, original magnification. (B) With long exposure times (e.g., 4 weeks), IGFBP-2 hybridization is detectable over discrete basophilic endometrial cells (arrows) near the circular muscle layer of the myometrium (cm); original magnification, 400×. (C) IGFBP-3 hybridization (blue-green grains) over endometrial stromal cells. Arrows point to IGFBP-3-positive endothelial cells; original magnification, 200×. (D) Periluminal transcription of

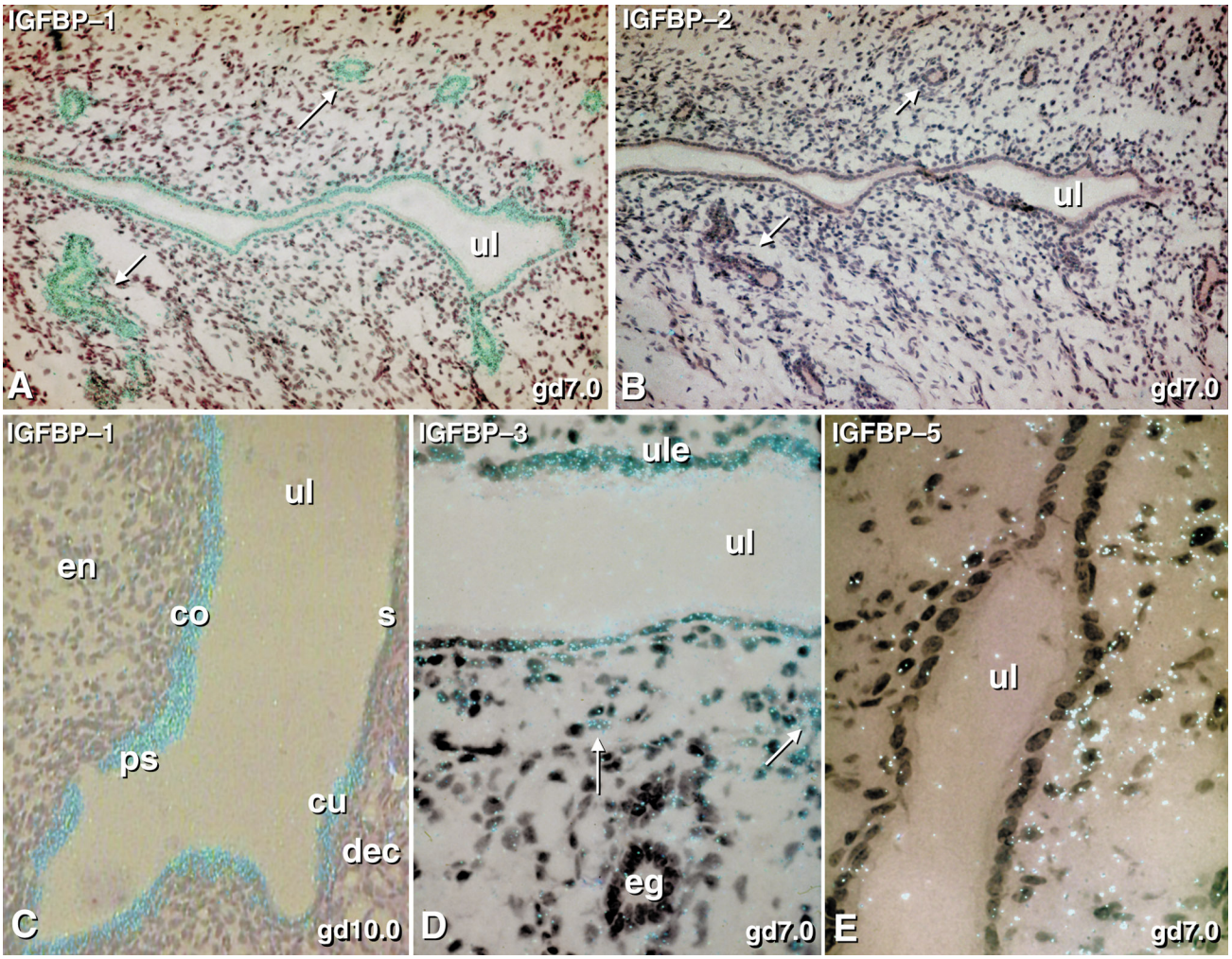


FIG. 2. IGFBP transcription by the secretory components of the postimplantation uterus. (A) High-power EP/BF photomicrograph shows IGFBP-1 hybridization (d7.0 pc) over columnar epithelial cells of uterine lumen (ul) and all endometrial glands (arrows); original magnification, 100×. (B) Adjacent section showing absence of IGFBP-2 hybridization in luminal and glandular environments. Glands in B are contiguous with those in A (arrows); 100×, original magnification. (C) As the decidua grows, luminal epithelial cells surrounding the decidua become squamous in appearance. High levels of IGFBP-1 hybridization are detected in columnar (co) and pseudostratified (ps) luminal epithelial cells surrounding the nondecidualized endometrium, with lower levels in the cuboidal (cu) and squamous (s) epithelia near the decidua (d10.0); original magnification, 200×. (D) IGFBP-3 mRNA is expressed in the luminal (ule), but not glandular, epithelium, as well as in some periluminal stromal cells at d7.0 (arrows); original magnification, 400×. (E) IGFBP-5 is expressed by periluminal stroma at d7.0; original magnification, 400×. Abbreviations: co, columnar epithelium; cu, cuboidal epithelium; dec, decidua; eg, endometrial gland; en, nondecidualized endometrium; ps, pseudostratified epithelium; s, squamous epithelium; ul, uterine lumen; ule, uterine luminal epithelium.

DXC-151A CCD color video camera (Sony Corp., Mahwah, NJ) connected to a PowerMacintosh 7100/80 AV computer (Apple Computer, Cupertino, CA). Software used for digitization and subsequent analysis included Apple Video Player (Apple Computer, Cupertino, CA), NIH Image (public domain software by Wayne Rasband, U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image/), and Adobe Photoshop (Adobe Corp., Mountain View, CA).

IGFBP-5 in the stroma. (E) IGFBP-6 mRNA is detected over connective tissue in the stratum vascularis (sv) of the myometrium after long exposure times; original magnification, 400×. (F) IGFR1 mRNA is widely expressed within the myometrium (particularly the circular layer) and the endometrium. Note also hybridization over an endometrial capillary (arrow); original magnification, 200×. Abbreviations: bv, blood vessel; cm, circular muscle; lm, longitudinal muscle; m, myometrium; sv, stratum vascularis; ul, uterine lumen.

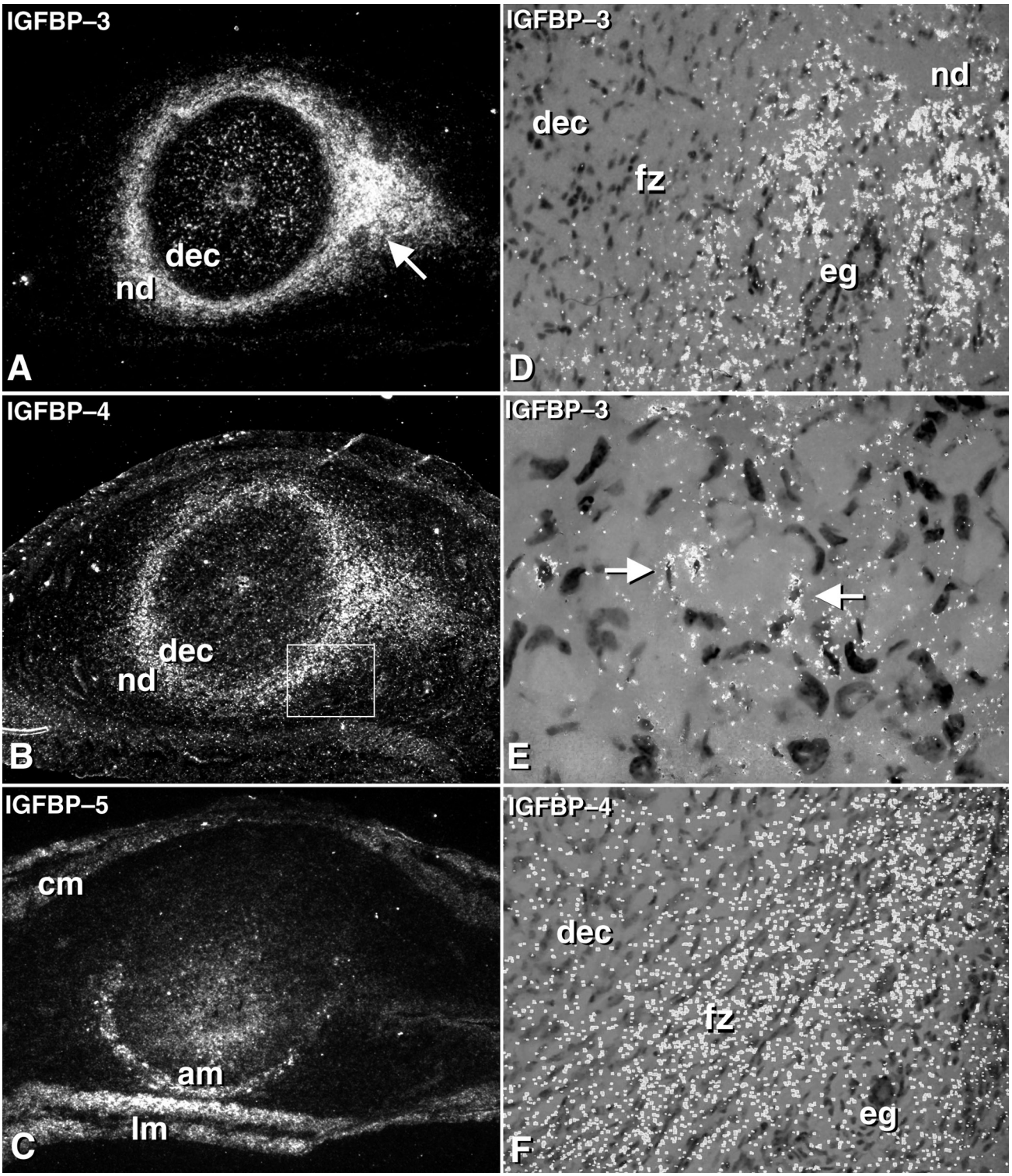


FIG. 3. Peridecidual IGFBP transcription on Gestational Day (gd) 7.0. (A) Low-power (80×, original magnification) dark-field (DF) photomicrograph of a d7.0 pc uterus showing IGFBP-3 hybridization over nondecidualized endometrial stromal cells (nd), particularly in periluminal area (large arrow). (B) Low-power DF photomicrograph of a nearby section hybridized with IGFBP-4 probe. Note diffuse pattern of expression over both decidualized (dec) and nondecidualized stroma; original magnification, 80×. (C) Narrow band of IGFBP-5 hybridization surrounding the decidua. Section is slightly oblique, and expression is highest at antimesometrial pole (am). Also note positive signal in both

RESULTS

The IGF binding proteins are present in the uterine environment throughout gestation. Changes in their expression patterns are associated with important events in pregnancy such as embryonic implantation, decidual growth, vascular remodeling of the endometrium, and preparation of the myometrium for parturition.

IGFBPs in the Preimplantation Uterus

As a positive control for this study, we confirmed the observations of Girvigian *et al.* (1994) in the cycling rat uterus by detecting late estrous transcription of IGFBP-2 at low levels in the ULE, IGFBP-3 in the periluminal stroma, and IGFBP-5 in both layers of the myometrium (data not shown). In addition, we detect several IGFBP mRNAs in preimplantation uteri (d3.5 pc). Surprisingly (see Discussion), IGFBP-1 hybridization is detected in the endometrial glands farthest from the uterine lumen, but is generally absent from the luminal epithelium and from glands near the lumen (Fig. 1A). At d3.5, IGFBP-3 mRNA is limited to a few scattered periluminal stromal cells, some of which are endothelia (Fig. 1C). Markoff *et al.* (1995) reported expression of IGFBP-4 by mouse periluminal stromal cells at d4, and we see similar hybridization in the d3.5 rat (not shown). In addition, we observe IGFBP-5 mRNA throughout the periluminal stroma, and also in both layers of the myometrium (Fig. 1D).

With comparatively long exposure times (e.g., 4 weeks), we also detect weak IGFBP-1 hybridization over a sparse number of stromal cells (not shown). Similarly, IGFBP-2 hybridization becomes detectable over small, scattered basophilic endometrial cells near the myometrium (Fig. 1B) and IGFBP-6 mRNA is observed in connective tissue of the myometrial stratum vascularis (Fig. 1E). Confirming the work of Kapur *et al.* (1992), we detect IGF-I transcription in d3.5 glandular and luminal epithelial cells, as well as stromal cells (not shown). Likewise, we extend the work of Zhou and Bondy (1992) to an earlier stage, detecting IGFR1 mRNA (Fig. 1F) over cells in both the endometrium and myometrium. IGFR1 hybridization is greatest over endometrial capillaries and the inner circular layer of the myometrium.

Uterine IGFBPs during Decidualization

We find a significant increase in the mRNA expression of several IGFBPs following implantation, extending to the cellular and transcriptional levels a report of increased IGFBP peptide levels in whole endometrial extracts at corresponding stages in pregnant mice (Markoff *et al.*, 1995). Taken together, these results suggest that IGFBPs can exert their activity at or close to their sites of synthesis. As early as 7 days pc the epithelial cells lining the uterine lumen and all endometrial glands strongly express IGFBP-1 mRNA (Fig. 2A). These two epithelial populations comprise the major secretory components of the rodent uterus. Interestingly, although IGFBP-2 is prominently expressed by the rat ULE during proestrus and estrus (Girvigian *et al.*, 1994), it is completely undetectable in these cells during pregnancy (Fig. 2B). As decidualization proceeds (e.g., d10.0), ULE IGFBP-1 mRNA levels remain high in the tall columnar and pseudostratified epithelium of the interembryonic regions, but hybridization is less intense, and is sometimes almost undetectable, in the cuboidal and particularly the squamous epithelia surrounding the decidua (Fig. 2C). Expression of IGFBP-1 (and IGFBP-5, see below) may account for some of the unidentified bands visible in ligand blotting experiments reported by *Markoff et al.* (1995). Low levels of IGFBP-4 transcription are also detected in some, but not all, endometrial glands (not shown), while in contrast, IGFBP-3 hybridization occurs in the ULE but not the glands (Fig. 2D).

After implantation, several IGFBPs, including IGFBPs -3 (Fig. 2D), -4, and -5 (Fig. 2E) are also expressed by nondecidualized stromal cells adjacent to the luminal epithelium. IGFBP-3 is particularly strongly expressed by periluminal stromal cells near the decidua (see heavy arrow, Fig. 3A).

The outer margin of the rat decidua is a thin, collagen-rich capsule of flattened, elongated fibroblasts termed the fibrous zone (Bridgman, 1948a). A laminar pattern of IGFBP mRNA expression is detected in cells on both sides of this margin, particularly surrounding the antimesometrial aspect of the rapidly proliferating secondary decidual zone (see Figs. 3 and 4). An intense ring of IGFBP-3 hybridization is seen over nondecidualized stromal cells surrounding the antimesometrial decidua beginning at d7.0 (Figs. 3A and 3D) and extending mesometrially by d11.5 (see Fig. 4C). This ring of IGFBP-3 is continuous with the hybridization over periluminal cells mentioned earlier, suggesting that IGFBP-3 may be buffering IGF communication between the lumen and the decidua. Likewise, a similar, albeit less extensive, ring of stromal IGFBP-5 expression is detectable from d7.0 (Fig. 3C) through d14.5 (Fig. 5F). IGFBP-4 mRNA expression occurs in a band straddling and including the

layers of the myometrium (cm, circular layer; lm, longitudinal layer); original magnification, 80×. (D) High-power EP/BF photomicrograph from same section as in A. Note the presence of endometrial glands, which are characteristic of nondecidualized tissue, within IGFBP-3-positive stroma; original magnification, 200×. (E) Expression of IGFBP-3 mRNA by discrete cells within the antimesometrial decidua. Arrows point to endothelia; original magnification, 400×. (F) High-power videomicrograph of area boxed in B. Diffuse IGFBP-4 hybridization is detected over cells on both sides of the decidual margin, particularly in the fibrous zone (fz). Note the absence of grains over an endometrial gland (eg); original magnification, 200×. Abbreviations: am, antimesometrial pole; cm, circular myometrium; dec, decidua; eg, endometrial gland; fz, fibrous zone; lm, longitudinal myometrium; nd, nondecidualized endometrium.

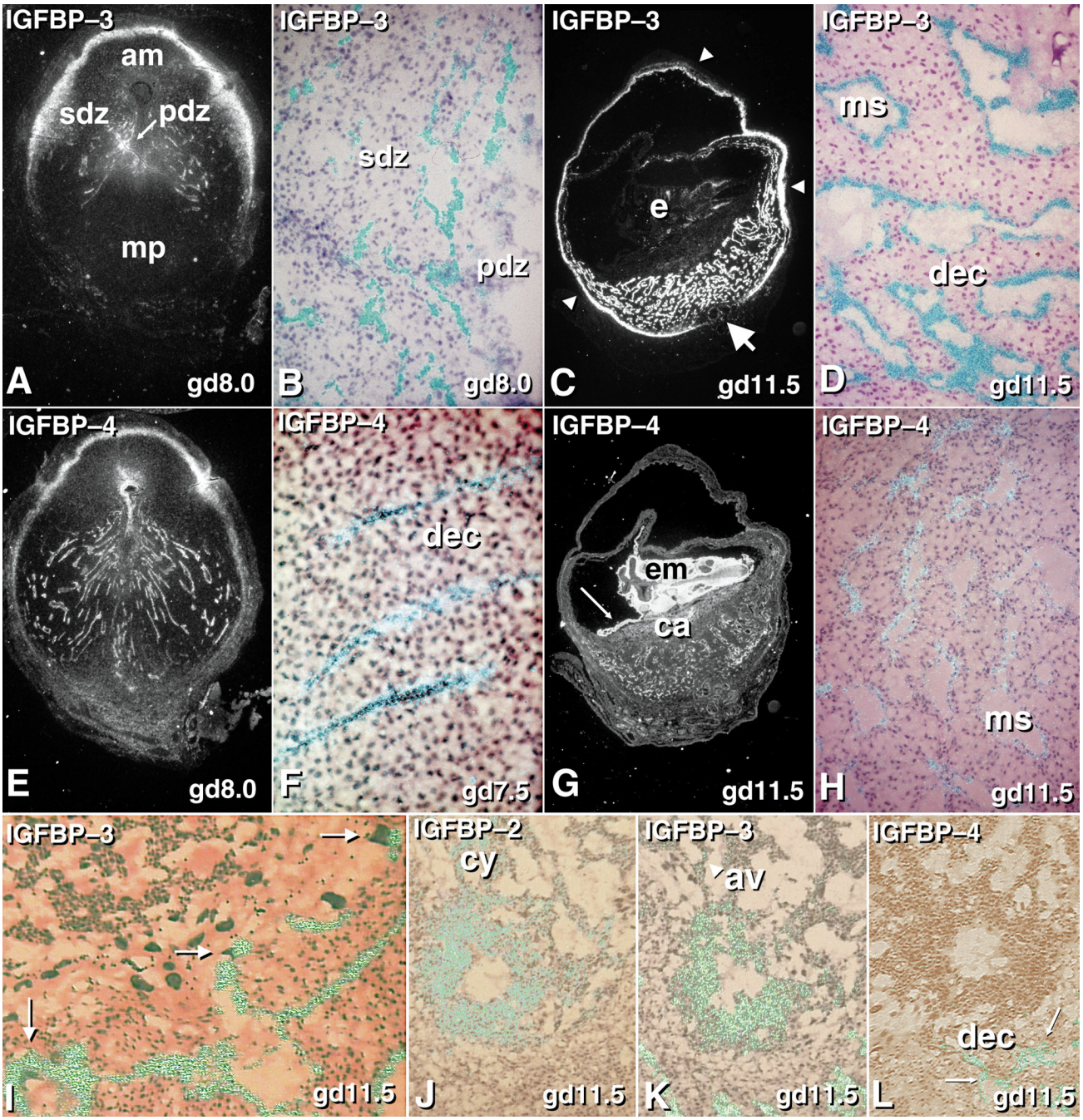


FIG. 4. IGFBPs in the decidual vasculature. A, C, E, and G are low-power DF photomicrographs at 80×, original magnification (A, E) or 60× (C, G). B, D, F, and H-L are high-power EP/BF photo or video micrographs at 200× magnification, except F, which is at 400×. (A and B) IGFBP-3 mRNA is expressed in capillary endothelial cells surrounding the d8.0 pc primary decidual zone. (C and D) IGFBP-3 transcripts are detected over all endothelial cells in dilated maternal blood sinuses at d11.5 pc. Note also the peridecidual IGFBP-3 hybridization (arrowheads), weaker signal in uterine spiral artery (large arrow), and expression in embryonic myotomes (e). (E) IGFBP-4 hybridization at d8.0 over early decidual blood vessels in the mesometrial pole. (F) Strong IGFBP-4 mRNA hybridization is detected over early decidual capillaries (d7.5). (G and H) The IGFBP-4 mRNA pattern is similar to, but weaker than, the pattern of IGFBP-3 expression shown in C and D. Note strong hybridization over the embryonic mesoderm (em) and yolk sac (arrow). (I) Trophoblast giant cells (arrows) are frequently seen adjacent to IGFBP-3-positive decidual endothelia at d11.5. Occasional giant cells are seen within the maternal sinusoids (see arrowed cell, lower left corner). (J) IGFBP-2 is strongly expressed by cytotrophoblasts (cy) in the region of the uterine central artery (d11.5). (K) Hybridization of IGFBP-3 over endothelial cells associated with the central artery, allantoic vessels (av, arrowhead), and decidua. (L) IGFBP-4 hybridization is limited to decidual vessels near the central artery (arrows). Abbreviations: am, antimesometrial pole; av, allantoic blood vessel; ca, chorioallantoic plate; cy, cytotrophoblasts; dec, decidua; e, embryo; em, embryonic mesoderm; mp, mesometrial pole; ms, maternal sinusoid; pdz, primary decidual zone; sdz, secondary decidual zone.

fibrous zone, encompassing both decidualized and nondecidualized cells (Figs. 3B and 3F). This band of IGFBP-4 transcription gradually diminishes until the placental stages, when it is undetectable (e.g., d14.5). In addition to the ring-like expression surrounding the decidua, individual stromal cells and capillaries deep within the antimesometrial capsule express IGFBP-3 mRNA at d7.0 (Fig. 3E).

As pregnancy proceeds, the decidua continues to grow and the uterine vasculature within this region is remodeled, largely through endothelial hypertrophy and vessel dilation (Welsh and Enders, 1991). IGFBP-3 hybridization is abundant at d8.0 in the lateral antimesometrial capillary plexus, which surrounds the thin, periembryonic primary decidual zone (Figs. 4A and 4B). In contrast, we detect IGFBP-4 mRNA in blood vessel endothelia throughout the decidua, particularly in the glycogenic mesometrial aspect (Figs. 4E and 4F). During this stage of vascularization we detect considerable IGFBP-4 hybridization in both decidual capillaries and uterine arteries. After the decidual capillary meshwork is laid down, maternal blood vessels close to the implantation site begin to dilate (Krehbiel, 1937; Welsh and Enders, 1991). This process gradually extends throughout the decidua until d11, when nearly all decidual vessels are sinusoidal. These sinusoids are the precursors of placental-stage lacunae through which maternal blood will flow. As dilation proceeds, endothelial IGFBP-3 mRNA increases, and IGFBP-4 mRNA decreases. By d11.5 very strong IGFBP-3 hybridization is detected over cells lining the sinusoids throughout the decidua (Figs. 4C and 4D), while lower levels of IGFBP-4 remain detectable in a similar pattern (Figs. 4G and 4H).

Trophoblast giant cells are frequently observed adjacent to IGFBP-3-positive maternal endothelia (Fig. 4I). These giant cells are in the process of penetrating maternal vessels (Fig. 4I), as well as phagocytosing dead or dying decidual cells (for review, see Hoffman and Wooding, 1993). Within the large uterine arteries, we detect IGFBP-3 and -4 mRNAs over endothelial cells, with IGFBP-3 also in the tunica adventitia at d11.5 (see also Zhou and Bondy, 1992). Interestingly, IGFBP-2 hybridization is prominent over the glycogenic cytotrophoblasts which begin to accumulate at the confluence of the uterine central artery and the chorioallantoic vasculature at d11.5 (Fig. 4J). It is noteworthy that mice unable to produce IGF-II have much diminished placentas when compared to normal mice and that the difference in size is attributed to much lower numbers of glycogen-producing cells in the mutant placentas (Lopez *et al.*, 1996). IGFBP-3 mRNA is also detected in this region (Fig. 4K), although it is not clear whether the cells in question are of maternal arterial or allantoic endothelial origin. In contrast, little or no IGFBP-4 hybridization is detected in the arterial region, except over decidual sinusoids (Fig. 4L).

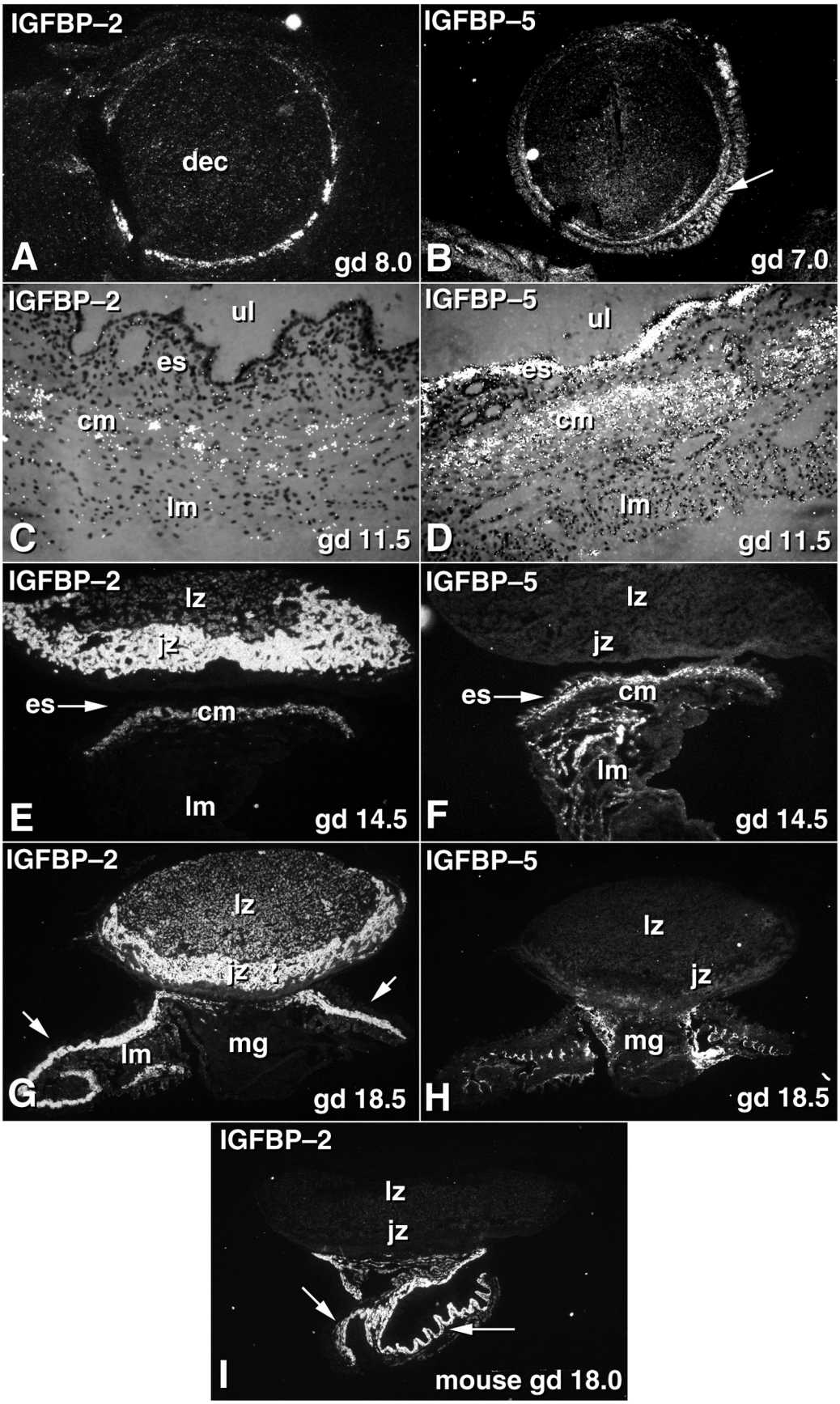
Like the endometrium, the myometrium undergoes a program of growth during pregnancy (Reynolds, 1965), and IGFBPs have been associated with muscle growth and differentiation *in vitro* (Ernst *et al.*, 1992; James *et al.*, 1993; Bach *et al.*, 1994; Ewton and Florini, 1995; Silverman *et al.*, 1995). Several IGFBP mRNAs are detected in uterine muscles during decidualization. At 8.0 days pc expression of IGFBP-2 mRNA is detectable in the inner aspect of the circular layer (Fig. 5A). IGFBP-5, present in the myometrium at pre-implantation stages, continues to be expressed by both the outer longitudinal and the inner circular muscle layers well after implantation (Fig. 5B). As decidualization proceeds, myometrial IGFBP-2 mRNA slowly increases in the circular layer (Fig. 5C), while IGFBP-5 transcription gradually decreases in the longitudinal layer until it is almost absent at d11.5 (Fig. 5D). IGFBP-6 hybridization is barely, if at all, detectable at d7.0 (Fig. 6A), although significant IGFBP-6 signal is observed in both myometrial layers by d11.5 pc (Fig. 6B).

IGFBPs in the Mid- to Late-Gestation Uterus and Placenta

Decidualization is essentially complete and placental structures are well differentiated by d11–12 pc in the rat (Everett, 1935; Krehbiel, 1937; Bridgman, 1948a,b). By this point, rapid growth of the embryo, the decidua, and the extraembryonic cavities has displaced the bulk of the nondecidualized glandular endometrium to the portions of the uterus between conceptuses. IGFBP-1 continues to be expressed by the epithelium of the uterine lumen throughout pregnancy (e.g., d18.5, Fig. 7A), although epithelial IGFBP-3 transcripts are no longer detectable (Fig. 7B). However, the periluminal stroma continues to express IGFBP-3 (Fig. 7B), IGFBP-4, and IGFBP-5 mRNAs through late gestation. In addition, periluminal IGFBP-6 transcription is detected by d18.5 (Fig. 7C).

There is little change in decidual IGFBP expression during the placental stages, save for the near disappearance of IGFBP-4 hybridization from decidual sinusoids (d18.5, not shown). In contrast, IGFBP-3 transcripts continue to be detected over the squamous linings of maternal lacunæ. Late in gestation, all that remains of the nondecidualized endometrium is a narrow strip of tissue beneath the decidua and in the regions between concepti. These nondecidualized stromal cells at the placental periphery continue to express substantial levels of IGFBP-3 mRNA and, to a lesser degree, IGFBP-5 mRNA at d18.5.

Myometrial growth continues into the placental period. By late gestation the uterus has developed considerable muscle mass, and changes in IGFBP expression indicate a possible role for the IGF system in myometrial anabolism. Relatively few myometrial cells are positive for IGFBP-2 hybridization at d11.5 (Fig. 5C), but by d14.5 substantial levels of IGFBP-2 mRNA are detected in the circular layer (Fig. 5E), and this continues through late gestation (Fig. 5G). In contrast, IGFBP-5 transcription at d14.5 is less extensive than that in decidual stages (Fig. 5F) and by d18.5 is limited to the connective tissue surrounding the longitudinal layer (Fig. 5H). Throughout pregnancy myometrial IGFBP-6 signals increase until d14.5, when very intense hybridization is detected in both muscle layers (Fig. 6C). This pattern


IGFBP–2
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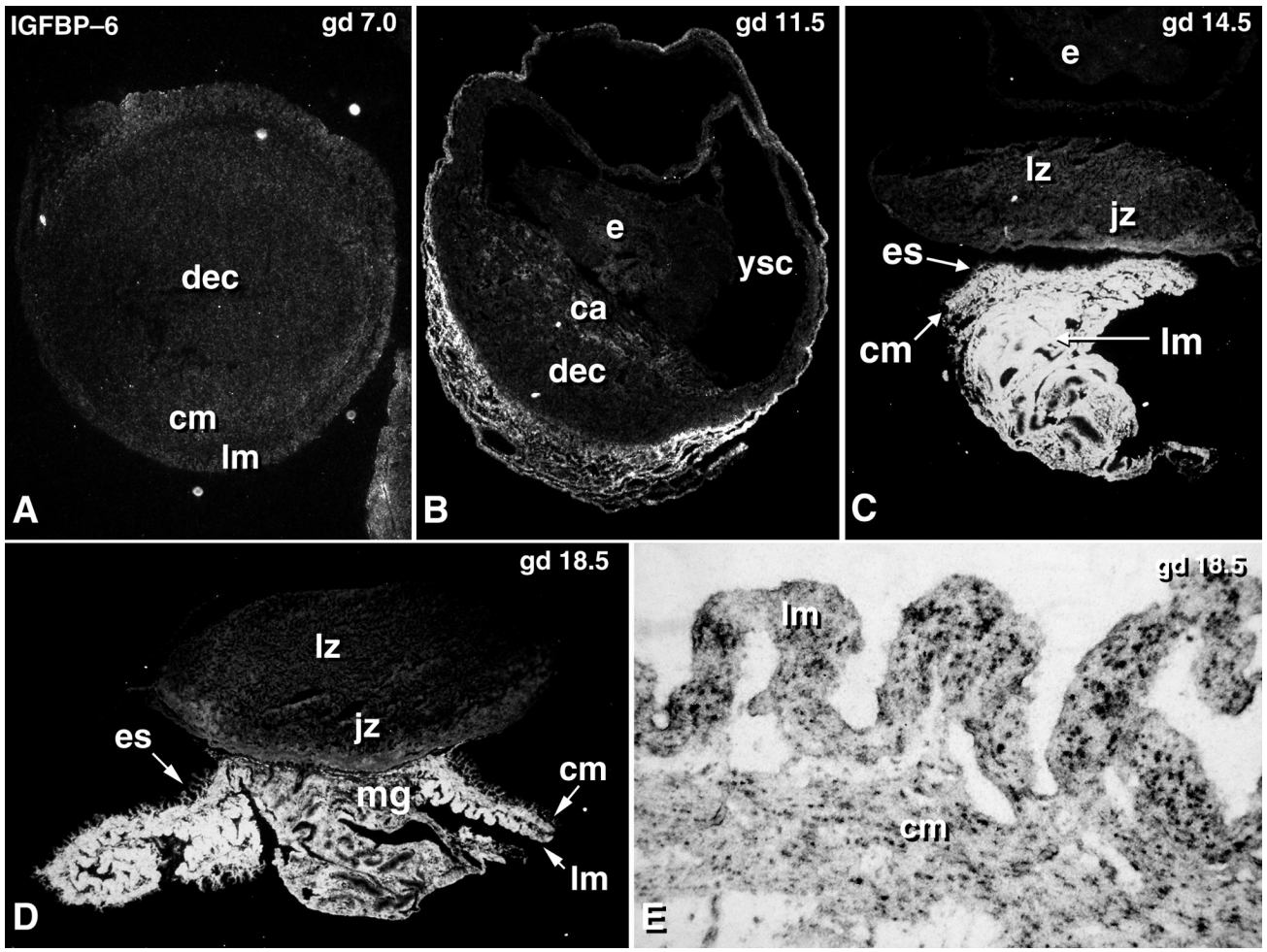

FIG. 6. IGFBP-6 is upregulated in uterine muscle during pregnancy. (A–D) Low-power dark-field photomicrographs. (A) Relatively little IGFBP-6 mRNA is detected at d7.0 pc; 120× (all magnifications are given at original sizes). (B) Both myometrial layers express IGFBP-6 at e11.5; 100×. IGFBP-6 is expressed at very high levels in the myometrium and endometrial stroma at d14.5 (C; 60×) and d18.5 (D; 60×). (E) Bright-field videomicrograph showing accumulation of IGFBP-6 mRNA over and around myometrial nuclei; 100×. Abbreviations: cm, circular muscle; dec, decidua; ca, chorionic plate/early placenta; e, embryo; es, endometrial stroma; lm, longitudinal layer; lz, labyrinthine zone of placenta; jz, junctional zone of placenta; mg, area of metrial gland.

FIG. 5. Myometrial expression of IGFBP-2 and -5 mRNAs from gd 8.0 to gd 18.5. (A) Low-power DF photomicrograph of IGFBP-2 hybridization over cells of the d8.0 pc inner circular muscle layer; original magnification, 100×. (B) Low-power DF photomicrograph of d7.0 pc uterus showing IGFBP-5 transcripts in both layers of the myometrium (arrow points to longitudinal layer); original magnification, 100×. (C) High-power (200×) EP/BF image of IGFBP-2 expression in inner layer of the d11.5 pc myometrium. (D) 200× EP/BF image of IGFBP-5 transcription in d11.5 pc uterine circular muscle layer and periluminal stroma (es). (E) High levels of IGFBP-2 mRNA expression in d14.5 circular myometrium (cm). No hybridization is detected in the adjacent periluminal stroma. Notice moderate IGFBP-2 expression in the labyrinthine placenta (lz) and extremely strong signal in junctional zone cytotrophoblasts (jz); original magnification, 80×. (F) IGFBP-5 transcription is nearly absent in the d14.5 circular muscle layer (cm, compare with D). Note hybridization in nondecidualized endometrial stroma (arrow) and connective tissue surrounding the longitudinal muscles; original magnification, 80×. (G) Circular muscle IGFBP-2 mRNA expression continues at e18.5; original magnification, 60×. (H) Myometrial IGFBP-5 hybridization is limited to superficial connective tissue layers of the longitudinal muscle at d18.5 pc (EP/BF); original magnification, 60×. (I) Near absence of IGFBP-2 hybridization in mouse junctional zone cytotrophoblasts (d18.0); original magnification, 60×. Note similarity of circular muscle signal to that of the rat (E). Abbreviations: cm, circular muscle; dec, decidua; es, endometrial stroma; jz, junctional zone of placenta; lm, longitudinal muscle; lz, longitudinal zone of placenta; mg, region of granulated metrial gland; ul, uterine lumen.

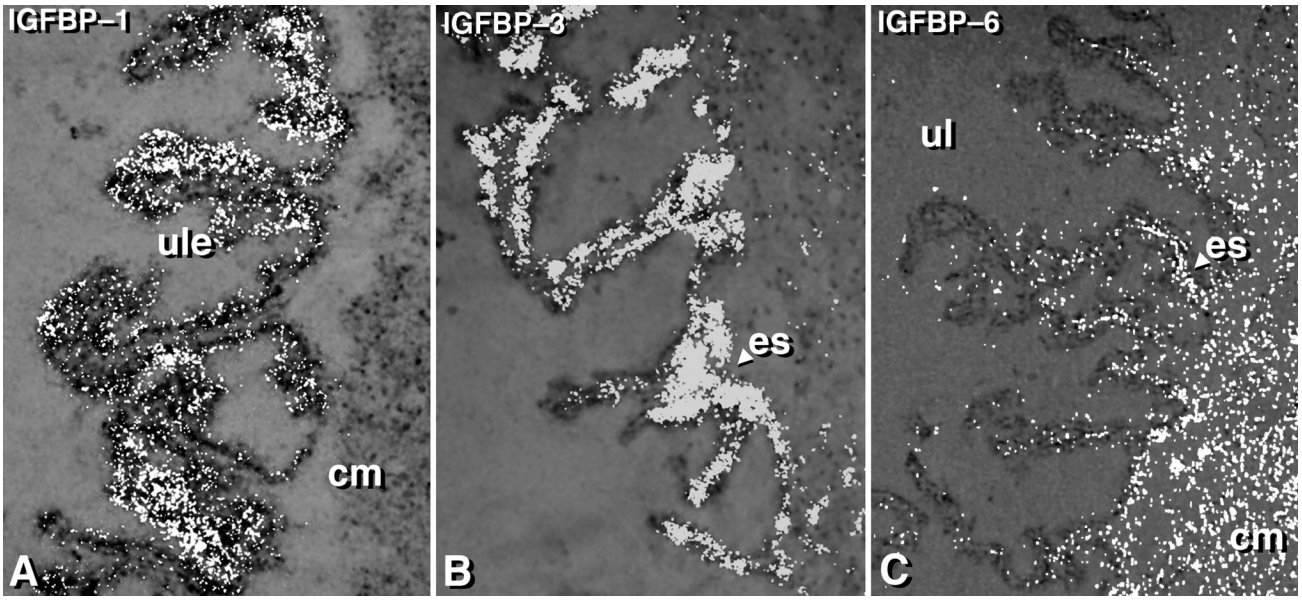

FIG. 7. Periluminal IGFBP expression during placental stages. (A) High-power EP/BF photomicrograph of IGFBP-1 hybridization in uterine luminal epithelial (ule) cells at d18.5 pc; 200× magnification (all magnifications given as original sizes). (B) Similar section showing IGFBP-3 transcript in periluminal endometrial stroma (es); 200×. (C) Bright-field video micrograph of IGFBP-6 mRNA in d18.5 stroma; 200×. Abbreviations: cm, circular muscle; es, endometrial stroma; ul, uterine lumen; ule, uterine luminal epithelial cells.

continues through d18.5 (Fig. 6D). IGFBP-6 hybridization is concentrated over and around myometrial nuclei during late gestation, consistent with Lobel *et al.*'s report (1965) of perinuclear accumulation of cytoplasmic RNAs in hypertrophic myometrial cells.

The placenta of the rat and mouse consists of splanchnopleure-derived blood vessels through which fetal blood flows (Davies and Glasser, 1968), surrounded by two layers of syncytiotrophoblasts and a fenestrated cytotrophoblast epithelium (Jollie, 1964; Enders, 1965; Kirby and Bradbury, 1965). These complexes, analogous to primate chorionic villi, form a labyrinthine zone which is bathed in maternal blood. Beneath the labyrinthine zone and adjacent to the decidua is the junctional zone, which marks the deepest widespread penetration of the trophoblast into maternal tissue and which is also bathed by maternal blood (Davies and Glasser, 1968). In agreement with Zhou and Bondy (1992), we detect moderately high levels of IGFBP-2 hybridization in labyrinthine trophoblast epithelia and very high levels over junctional zone cytotrophoblasts in mid- to late-gestation rat placentas (Figs. 5E and 5G). Surprisingly, only very weak hybridization of IGFBP-2 probe was detectable over cytotrophoblasts in the mouse placenta (Fig. 5I). In our preparations, the myometrium was included on placental sections, and comparable levels of myometrial IGFBP-2 mRNA were detected in both rat and mouse (Figs. 5G and 5I), arguing that the diminished hybridization in cytotrophoblasts represents a true difference in tissue-specific gene expression between these closely related species.

The yolk sac performs critical placental functions throughout pregnancy (Everett, 1935) and is the initial site of hematopoeisis within the conceptus (Moore and Metcalf, 1970). Confirming and extending the observation of Zhou and Bondy (1992) that IGFBP-1 (Fig. 8A) is expressed by the yolk sac epithelium (YSE) throughout gestation and that IGFBP-4 (Fig. 8C) is expressed by the YSE during mid-gestation (d11.5–d14.5), we in addition detect epithelial IGFBP-2 hybridization at d11.5 (Fig. 8B). YSE hybridization of IGFBP-2 declines after d14.5 pc and is not detectable at d18.5 (not shown), similar to the decline in epithelial IGFBP-4 expression reported by Zhou and Bondy (1992). These changes in binding protein expression coincide with, and may be related to, increases in the transport of macromolecules (e.g., maternal antibodies, Padykula *et al.*, 1966) and glycogen storage (Bridgman, 1948a) by the visceral yolk sac epithelium. Interestingly, these physiological changes occur as the parietal endoderm degenerates (Padykula *et al.*, 1966), exposing the visceral YSE to the contents of the uterine lumen, which may include maternal IGF-I and IGFBPs. Meanwhile, the yolk sac vasculature continues to express both IGFBP-3 and IGFBP-4 mRNAs throughout the placental stages (d14.0–18.0) in the mouse (Figs. 8D and 8E, respectively) and the rat (Zhou and Bondy, 1992). IGFBP-1 and IGFBP-4 mRNAs are also expressed by cells of the amniotic membrane at d14.5 and d18.5 pc (not shown).

DISCUSSION

The pregnant rodent uterus is a remarkably plastic system which undergoes a program of rapid growth and differentiation

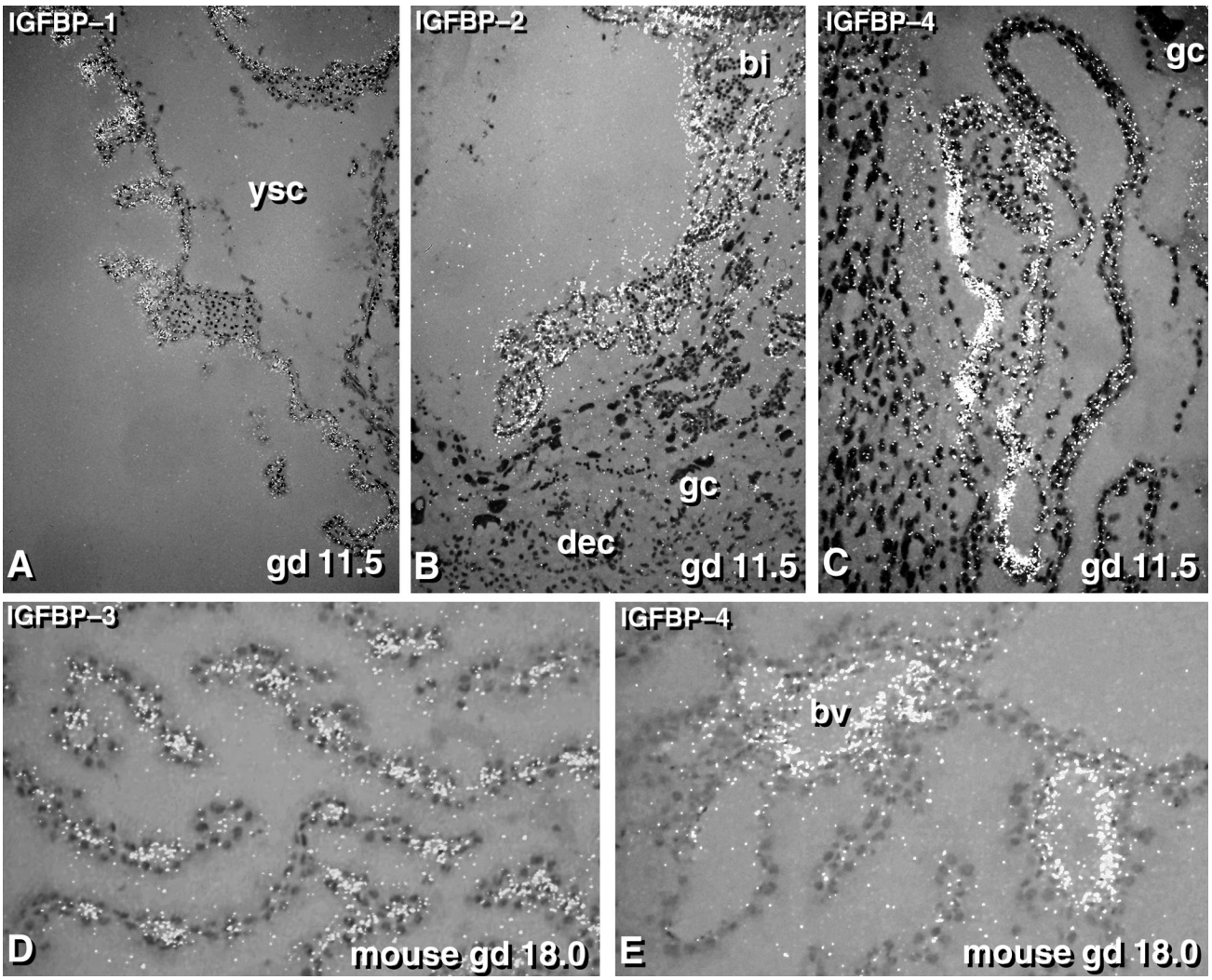

FIG. 8. Expression of IGFBPs in the yolk sac membranes. EP/BF photomicrographs showing (A) IGFBP-1 transcription in the rat yolk sac epithelium at d11.5 pc, (B) IGFBP-2 hybridization in the yolk sac epithelium at d11.5, and (C) IGFBP-4 signal in the yolk sac epithelium at d11.5. (D) IGFBP-3 and (E) IGFBP-4 expression in the d18.0 mouse yolk sac blood vessel endothelia. A, B, and C are at 200× original magnification; D and E are at 400× original magnification. Abbreviations: bi, blood island; bv, blood vessel; gc, trophoblast giant cell; ysc, yolk sac cavity.

in order to support an embryo and then rapidly resets itself after parturition. Previous studies have implicated the IGF system in this process (for review, see Simmen *et al.*, 1995), but the specific roles of IGF binding proteins remain elusive. To address this issue we have examined uteroplacental IGFBP mRNA expression patterns throughout pregnancy, and we find that all IGFBPs are present in this environment, with unique temporal and spatial relationships. Previous work has elucidated the cellular pattern of IGFBP mRNA expression in the cycling, nonpregnant rat uterus (Girvigian *et al.*, 1994). While there are some similarities in IGFBP expression between diestrous and pregnant preimplantation uteri, there are also significant differences. The cellular sites of IGFBP transcription described here correlate well with IGFBP peptide profiles from homogenized pregnant mouse endometria examined by Markoff *et al.* (1995). It is therefore likely that IGFBP peptides function in an autocrine or paracrine manner, i.e., close to their sites of synthesis. Taken together, these patterns suggest that the IGFBPs play roles in processes as diverse as supporting implantation, limiting decidualization, modulating local levels of vascular IGFs, and regulating uterine muscular growth.

Implantation

Early conceptuses enter the uterine environment shortly after fertilization, and this environment is rich in

secretory products derived from the luminal epithilium and the contiguous endometrial glands. At least one of these products, leukemia inhibitory factor, is required for normal implantation in mice (Stewart *et al.*, 1992). It is with interest, then, that we note the strong expression of IGFBP-1 transcription in preimplantation endometrial glands at d3.5 pc, followed by expression in all secretory epithelia by d7.0. This finding extends both to the transcriptional level and to an earlier stage, the work of Sadek *et al.* (1994), which demonstrated the presence of IGFBP-1 peptide in some, but not all, endometrial glands at d5.0 and nearly all glands and the ULE at d7.0. Since IGFBP-1 peptide was not detectable at d3.0, it is likely that transcription at d3.5 represents the onset of IGFBP-1 gene expression. Further, IGFBP-1 has been proposed to act primarily as an autocrine/paracrine factor in the uterus (Sadek *et al.*, 1994), and colocalization of transcript and peptide supports this notion.

Our results validate the detection of epithelial IGFBP-1 transcription in artificially induced deciduomata (Croze *et al.*, 1990). Croze *et al.*'s observation (1990) coupled with the well-characterized expression of IGFBP-1 by primate decidual cells *in vivo* (Bell *et al.*, 1985; Fazleabas *et al.*, 1989; Rutanen *et al.*, 1991) has led to the conclusion that IGFBP-1 is intimately related to decidualization and may even be a molecular marker for this event (Rutanen *et al.*, 1991). However, decidualization is a directional process beginning at the luminal surface of the uterus and progressing toward the myometrium (Krehbiel, 1937). At d3.5, the IGFBP-1-positive glands are generally those farthest from the lumen, suggesting instead that IGFBP-1 is associated with *pre*decidual transformation of the uterus and, at least in the rodent, may be more properly regarded as a more generalized pregnancy-associated peptide than as a decidual factor.

IGFBPs -1 and -2 both contain an RGD peptide sequence, which is associated with binding to the extracellular matrix. Recently, IGFBP-1 has been shown to localize to the extracellular matrix by binding to $\alpha5\beta1$-integrin and to enhance the migration of CHO fibroblasts (Jones *et al.*, 1993a) and human cytotrophoblasts (Irving and Lala, 1995) on $\alpha5\beta1$-integrin-containing substrates. Notably, $\alpha5\beta1$-integrin is expressed by the rat uterine epithelium (Nishida *et al.*, 1991) and murine ectoplacental cone cells (Sutherland *et al.*, 1993), and antibodies against the $\beta1$-subunit significantly impair the ability of murine cytotrophoblasts to spread over decidual cells (Yoshimura *et al.*, 1995). We suggest that IGFBP-1 may facilitate embryonic invasion of the ULE and decidua through a direct interaction with $\alpha5\beta1$-integrin. The other major ligand of $\alpha5\beta1$-integrin is fibronectin, and Damsky *et al.* (1994) have shown that blocking fibronectin binding accelerates cytotrophoblast invasion *in vitro*. IGFBP-1 may therefore act to promote cell motility by interfering with fibronectin–integrin binding.

In contrast to IGFBP-1, we detect no transcription of RGD-containing IGFBP-2 in the pregnant ULE. This is a dramatic difference from the cycling ULE, which transcribes copious amounts of IGFBP-2, particularly during proestrus (Girvigian *et al.*, 1994). Interestingly, IGFBP-2 strongly inhibits the migration-promoting activity of both IGFs on smooth muscle cells *in vitro* (Gockerman *et al.*, 1995). Blastocysts must migrate over and between ULE cells in order to implant succesfully; thus, loss of IGFBP-2 expression followed by the onset IGFBP-1 expression in the ULE may be a critical step in implantation at the biochemical level.

After penetrating the ULE, trophoblast cells are confronted with the endometrial stroma, where a complicated scheme of IGF buffering appears to be in place. IGFBP-5, which has been shown to enhance IGF activity when bound to cell surfaces (Jones *et al.*, 1993b), is prominently expressed by the periluminal stroma, particularly prior to implantation. Periluminal expression of IGFBP-5, coupled with IGF-I expression by the adjacent ULE (Kapur *et al.*, 1992), may provide a favorable environment for blastocyst growth.

Recent work has indicated that IGFBP-3 may act primarily as an inhibitor of mitogenesis (Valentinis *et al.*, 1995), and IGFBP-4 is generally a very potent inhibitor of IGF action (Jones and Clemmons, 1995). Periluminal hybridization to these mRNAs is most prominent over stromal cells in the regions between implantation sites and is very low or absent in the decidual zones. This is consistent with a role for these binding proteins having a negative effect on implantation, perhaps regulating the number of succesful implantations in a pregnancy.

Decidualization

The balance between IGF-induced proliferation or invasion and the activity of IGF inhibitors may be critical in normal pregnancy. Although surprisingly little is known with certainty about the details of decidual function (Tarachand, 1986; Abrahamsohn and Zorn, 1993), one of the most likely functions is to limit the scope of trophoblast invasion, a role which must be balanced against the need to support the respiratory needs of the conceptus. While implantation is clearly important to life, the aggressiveness of trophoblast invasion has been likened to a pseudomalignancy (Kirby and Cowell, 1968) and can result in highly metastatic choriocarcinomas (Strickland and Richards, 1992). The IGF system is thought to be important in the behavior of many malignancies (reviewed in LeRoith *et al.*, 1995a), and the uterine pattern of IGFBP hybridization is consistent with regulating trophoblast and decidual exposure to IGFs. As demonstrated in the ULE and periluminal stroma, uterine IGFBP expression occurs in layers, and this is also true with respect to the decidua. With the exception of blood vessels and a few IGFBP-3-expressing stromal cells, there is relatively little decidual IGFBP expression near the embryo. However, at the margins of the decidualized endometrium several IGFBPs are strongly expressed in a laminar array, with IGFBP-4 straddling the decidual boundary dur-

ing early decidualization, considerable IGFBP-3 transcription on the nondecidualized side of the boundary, and similar, but more limited, nondecidual IGFBP-5 hybridization. Taken together these observations suggest that a ''tug of war'' involving the IGF system may take place between invasive/mitogenic tissues and the relatively static nondecidualized endometrium.

Uterine Circulatory System

IGFBPs are thought to be crucial to the maintainence of circulating IGF levels and to the delivery of IGFs to particular tissues (reviewed in Delafontaine, 1995). We detect strong expression of two of the binding proteins, IGFBPs -3 and -4, in decidual blood vessels. In the adult, IGFBP-3 is the major serum IGFBP and can significantly extend the half-life of serum IGFs (Guler *et al.*, 1989). While uterine endothelial IGFBP-3 possibly contributes to the stability of circulating IGFs, it may also act in an autocrine fashion by binding to endothelial cell surfaces and modulating local IGF activity. During decidualization, maternal blood vessels are transformed into sinusoids, as endothelial cells hypertrophy and vessel walls become fenestrated. Hybridization of IGFBP-3 to decidual endothelia is detected throughout the sinusoidal transformation, peaking as trophoblast giant cells come into contact with maternal vessels. Likewise, IGFBP-3 mRNA expression is associated with development of blood vessels in the rat embryo and in several tumor types (J. Cerro, A. Grewal, and J. Pintar, unpublished results), so it is likely that IGFBP-3 is involved in the remodeling of the decidual vasculature.

Although IGFBP-4 is generally an IGF inhibitor (Jones and Clemmons, 1995), its expression in the vasculature has been associated with transport of IGFs across endothelia (Bar *et al.*, 1990). Interestingly, strong vascular IGFBP-4 expression coincides with rapid decidual mitogenesis, consistent with a role for IGFBP-4 in transporting IGF-I from the circulation to the proliferating decidua.

Uterine Musculature

Evidence has accumulated that the IGF system is important in muscle growth and differentiation, both *in vitro* (Dodson *et al.*, 1985; Florini *et al.*, 1991a,b; Bach *et al.*, 1994; Ewton and Florini, 1995) and *in vivo* (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). IGFBP-5 and IGFBP-6 are both prominently expressed in the estrous rodent myometrium, with IGFBP-5 peaking in diestrous muscle (Girvigian *et al.*, 1994). IGFBP-5 is associated with myoblast differentiation *in vitro* (Tollefsen *et al.*, 1989; Ewton and Florini, 1995), and high levels of IGFBP-5 transcription have been observed *in vivo* in developing embryonic muscles (Cerro *et al.*, 1993; Schuller *et al.*, 1993; Green *et al.*, 1994). Here we have determined that IGFBP-5 is expressed by both layers of the myometrium early in pregnancy, peaks at mid-gestation, and then becomes very limited in expression by the placental stages, suggesting that it functions in the myometrial proliferation of early postimplantation pregnancy. In contrast, IGFBP-2 is limited to the circular layer and reaches its highest level late in gestation. The functional significance of differential gene expression between the two myometrial layers is unclear, although this phenomenon is not uncommon [e.g., TGF-β3 (Das *et al.*, 1992) and tenascin (Julian *et al.*, 1994)]. A third pattern of myometrial expression occurs with IGFBP-6 mRNA, which gradually increases in both muscle layers until late in gestation, when hybridization signals are very intense. The rat IGFBP-6 promoter contains an estrogen receptor binding site (Zhu *et al.*, 1993), and uterine IGF-I is known to be estrogen responsive (Sahlin *et al.*, 1994), so it is likely that IGF-I and IGFBP-6 are coregulated by estrogen in the myometrium. Further, the extremely high levels of IGFBP-6 transcription during the placental period suggest that it may be the major modulator of IGF activity in the uterine muscle.

In addition to the complicated changes in uteroplacental IGFBP transcription described here, other groups have reported pregnancy-associated IGFBP-specific proteases in maternal serum (Davenport *et al.*, 1990; Giudice *et al.*, 1990; Hossenlopp *et al.*, 1990). Taken together, these results suggest the importance of precise IGFBP regulation during pregnancy. It remains unclear, however, whether the IGFBPs act primarily as IGF enhancers or inhibitors, or possibly as IGF-independent factors during pregnancy. It is perhaps most likely that the IGFBPs may do all of the above as a group, but that the individual IGFBPs have discrete, tissue-specific functions. A more precise answer requires better *in vivo* models, such as mice incapable of producing particular IGFBPs obtained through gene targeting (Pintar *et al.*, 1996).

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